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RAPID PUBLICATION

CONGENITAL CENTRAL HYPOVENTILATION SYNDROME: MUTATION ANALYSIS OF THE RECEPTOR TYROSINE KINASE RET

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Congenital central hypoventilation syndrome (CCHS) usually occurs as an isolated phenotype. However, 16% of the index cases are also affected with Hirschsprung disease (HSCR). Complex segregation analysis suggests that CCHS is familial and has the same inheritance pattern with or without HSCR. We postulate that alteration of normal function of the receptor tyrosine kinase, RET, may contribute to CCHS based on RET's expression pattern and the identification of RET mutations in HSCR patients. To further explore the nature of the inheritance of CCHS, we have undertaken two main routes of investigation: cytogenetic analysis and mutation detection. Cytogenetic analysis of metaphase chromosomes showed normal karyotypes in 13 of the 14 evaluated index cases; one index case carried a familial pericentric inversion on chromosome 2. Mutation analysis showed no sequence changes unique to index cases, as compared to control individuals, and as studied by single strand conformational polymorphism (SSCP) analysis of the coding region of RET. We conclude that point mutations in the RET coding region cannot account for a substantial fraction of CCHS in this patient population, and that other candidate genes involved in neural crest cell differentiation and development must be considered.

KEY WORDS: Congenital central hypoventilation syndrome, Hirschsprung disease, RET receptor tyrosine kinase, mutation detection

INTRODUCTION

Idiopathic congenital central hypoventilation syndrome (CCHS) is a rare disorder which results from an abnormality in the control of respiration [Coleman et al., 1980; Deonna et al., 1974; Fleming et al., 1980; Guilleminault et al., 1982; Haddad et al., 1978; Hunt et al., 1978; Mellins et al., 1970; Shannon et al., 1976; Weese-Mayer et al., 1992; Wells et al., 1980]. Patients typically present in the first hours of life with cyanosis and increased carbon dioxide during sleep, and in severe cases during sleep and

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wakefulness. CCHS has been associated with a variety of disorders including eye abnormalities [Haddad et al., 1978; Weese-Mayer et al., 1992], lack of heart rate variability [Haddad et al., 1978; Weese-Mayer et al., 1992; Woo et al., 1992], neuroblastoma [Bower and Adkins, 1980], ganglioneuroma [Swaminathan et al., 1989], and, most frequently, Hirschsprung disease (HSCR) [Bower and Adkins, 1980; Guilleminault et al., 1982; Haddad et al., 1978; Hamilton and Bodurtha, 1989; Minutillo et al., 1989;, O'Dell et al., 1987; Stern et al., 1981; Weese-Mayer et al., 1988; 1992; 1993] . Of note, neuroblastoma, ganglioneuroma, and HSCR have all been described as neurocristopathies, that is, disorders due to aberrations in neural crest cell migration, proliferation, or differentiation [Bolande, 1974]. It has been suggested that CCHS may share a common etiology with the other associated anomalies described as neurocristopathies [Bower and Adkins, 1980], and thus, may arise from failure of migration or differentiation of neural crest cells in early embryogenesis.

Familiality has been observed in CCHS. Segregation analysis established that the inheritance of CCHS can be explained by either multifactorial threshold or major locus models [Weese-Mayer et al., 1993]. Segregation analysis has also demonstrated HSCR to be a heterogeneous genetic disorder with multigenic, autosomal recessive, and autosomal dominant forms [Badner et al., 1990]. Although the molecular basis of CCHS is unknown, molecular genetic studies have identified two genes in humans which can confer genetic predisposition to HSCR. The first gene, HSCR1, was initially mapped to chromosome 10(q11.1) by linkage analysis [Angrist et al., 1993; Lyonnet et al., 1993] prior to the discovery of mutations in the receptor tyrosine kinase RET in sporadic and familial cases of HSCR [Angrist et al., 1995; Edery et al., 1994; Romeo et al., 1994]. More recently, a second gene, HSCR2, was mapped to 13q22 in a large, inbred Mennonite kindred [Puffenberger et al., 1994a] and subsequently a mutation in the G-protein coupled receptor, endothelin-B receptor (ET_B) gene, EDNRB, was identified [Puffenberger et al., 1994b]. Studies in the mouse demonstrated that targeted disruption of either the mouse ET_B, or one of its ligands, endothelin-3 (ET-3), results in a mutant megacolon phenotype [Baynash et al., 1994; Hosoda et al., 1994]. The genes that have been implicated in HSCR are important for the migration of cells originating from the neural crest and their subsequent development as enteric ganglia, possibly acting in the signaling pathway which differentiates pre-ganglion cells into maturity.

In children with CCHS HSCR occurs with a high incidence (16%) [Weese-Mayer et al., 1992]. Given that HSCR is rare, and that genetic mutations underly its molecular basis, we postulate that the co-occurrence of CCHS and HSCR arises from a common genetic basis. The RET gene is an appealing candidate for several reasons. First, RET plays a crucial role in cellular growth and transformation [Takahashi et al., 1985] and is clearly important for normal embryonic development [Schuchardt et al., 1994]. Second, gene expression studies in the mouse have documented RET expression on day 8.5 of embryogenesis in cell lineages of both the peripheral and CNS, and later expression in specific regions of the PNS, enteric nervous system and CNS [Pachnis et al., 1993]. Third, RET mutations have been identified in patients with neurocristopathies such as HSCR, and multiple endocrine neoplasia type 2A (MEN 2A) [Donis-Keller et al., 1993; Mulligan et al., 1993, 1994] and type 2B (MEN2B) [Carlson et al., 1994; Eng et al., 1994; Hofstra et al., 1994]. Finally, RET expression is specifically detected and increased in neuroblastoma cell lines [Itoh et al., 1992]. In order to evaluate RET as a candidate, we have conducted a mutation screen of the 20 RET exons which comprise the coding region of the gene. Additionally, in order to uncover other loci, we have searched for cytogenetic aberrations that would indicate a particular chromosomal region relevant to CCHS.

METHODS

Patient population and controls. Children were recruited who fulfilled the rigid criteria of alveolar hypoventilation in the absence of a primary pulmonary, neuromuscular, or focal brainstem

abnormality that might account for the hypoventilation. The diagnosis of idiopathic congenital central hypoventilation syndrome in each index case was confirmed in the polysomnography laboratory at Rush Children's Hospital (Chicago, Illinois). Blood samples were collected from each index case, parents of each index case, and sibs of each index case under informed consent. Samples from 14 index cases, 23 parents, and 16 sibs were collected. Ten of the index cases had isolated CCHS and 4 of the index cases had HSCR and CCHS. None of the parents or sibs had either CCHS or HSCR. We have previously reporteded 6 of the index cases with isolated CCHS and 3 of the index cases with HSCR and CCHS [Weese-Mayer et al., 1992; 1993]. The study was approved by the Human Investigation Committee at Rush-Presbyterian-St. Luke's Medical Center.

Control samples were 14 phenotypically normal individuals identified as members (parents) of the Centre d'Etude du Polymorphisme Humain (CEPH) reference families [Dausset, 1986].

DNA isolation. DNA was isolated by either a "salting-out" procedure [Miller et al., 1988] performed manually, or by an automated phenol-chloroform extraction protocol as performed by the Autogen 540 (Integrated Separation Systems, Natick, MA).

PCR and primers. Primer sequences used to amplify all 20 RET exons were obtained from previously published sources or designed using the Macintosh program Oligo, Version 4.0 based on intronic nucleotide sequence [Ceccherini et al., 1993; 1994; Takahashi et al., 1988; 1989; Kwok et al., 1993]. PCR analysis was carried out as in Angrist et al. [1993]. All primers were synthesized by Research Genetics (Huntsville, AL).

Single strand conformational polymorphism analysis (SSCP). For SSCP analysis [Orita et al., 1988; 1989], double stranded non-radiolabeled PCR products were denatured to single stranded (ss) DNA by incubating at 95°C for three minutes and then immediately placed on ice. In order to optimize the sensitivity of SSCP, average PCR product size of RET exons was 200 bases. All SSCP analyses were carried out using the Phast System apparatus (Pharmacia Uppsala, Sweden) which affords semi-automation and temperature control for optimization of SSCP analysis. The apparatus requires approximately 0.8 µl of each denatured sample to be loaded onto combs and then electrophoresed on a non-denaturing polyacrylamide gel at user-specified temperatures (4°C or 15°C in this case) for an appropriate number of volt-hours corresponding to the size of the PCR fragment. The gels were then fixed and silver-stained according to the manufacturer's protocol.

Cytogenetics. Metaphase chromosomes were prepared from lymphocytes according to a previously described harvest technique [Moorhead et al., 1960]. G-banding of the metaphase chromosome spreads was also performed according to a previously described banding technique [Seabright, 1971].

RESULTS

Single strand conformational Polymorphism (SSCP) of RET gene. SSCP analysis of the PCR products generated with primers specific for each of the 20 exons of the RET gene in the 14 index cases demonstrated no variants indicative of sequence changes unique to the index cases as compared to control individuals. Conformational variants were observed in exon 7 in three index cases; in exon 11 in two index cases; in exon 13 in two index cases; and in exon 15 in one index case. However, the same variants were also observed in control individuals and reported previously as polymorphisms in the RET gene [Ceccherini et al., 1994]. The polymorphisms detected represent a nucleotide change in alanine at position 432 in exon 7 with a reported allele frequency of 0.29. Another polymorphism was observed in exon 11, indicating a glycine to serine change at position 691 with an allele frequency of 0.79. A third nucleotide change in leucine at position 769 in exon 13 was observed and has a reported allele frequency of 0.74. A fourth polymorphism representing a nucleotide change in Serine at position

904 was reported with an allele frequency of 0.21 [Ceccherini et al., 1994].

Cytogenetic analysis. Samples from 14 index cases were karyotyped. All but one of the study subjects had a normal karyotype. One index case with CCHS and HSCR carried a familial pericentric inversion of chromosome 2; 46,XY,inv(2)(p11.2q13).

DISCUSSION

In this study we have sought to provide a molecular explanation for a putative defect in the respiratory control center of the brain leading to CCHS. Gross pathological abnormalities, or lesions have not been identified in CCHS patients, so it is likely that the defect leading to lack of respiratory control in CCHS patients is at the molecular level. It was postulated previously that CCHS has a genetic origin given its occurrence in identical twins [Khalifa et al., 1988] and association with HSCR in sisters [Haddad et al., 1978] and half-sibs [Hamilton and Bodurtha, 1989]. Moreover, the existence of genetic factors among CCHS index cases was demonstrated by a complex segregation analysis using a unified mixed model [Weese-Mayer, et al., 1993]. The fact that neurocristopathies such as HSCR, ganglioneuromas and neuroblastomas occur in CCHS points to genes involved in neural crest cell migration/differentiation/proliferation as likely candidates. We have studied the receptor tyrosine kinase RET for a mutation or sequence alteration which could be a contributing factor for CCHS and the association of CCHS with neurocristopathies. No such mutation has been identified after evaluating 20 exons of the RET gene on chromosome 10q11.2 in CCHS patients.

Both the expression pattern of RET and the involvement of RET mutations in associated disorders such as HSCR and MEN 2B (in which ganglioneuromas occur) has led us to consider that mutations in RET may be a common causative factor in CCHS. However, expression of RET in the CNS is most intriguing in the context of CCHS. The regions of the brain expressing RET in the CNS include the spinal cord and motor neurons of the hindbrain [Pachnis et al., 1993]. The hindbrain of the embryo differentiates into structures which give rise to the cerebellum, pons, and medulla oblongata. This hindbrain region is particularly important when considering CCHS because the neural system responsible for autonomic regulation of respiration is located in the medulla and pons, while the efferent output from this system to respiratory motor neurons is located in the cervical spinal cord. Thus, RET is expressed during development in a region of the brain in which the defect responsible for CCHS is thought to occur.

The fact that RET mutations have not been identified in this study is not simply explained by a failure to detect mutations by SSCP analysis. Although detection rate estimates for SSCP have been reported to be 35% to 100% [Cotton, 1993], the Phast System apparatus used in SSCP protocols in this study was recently estimated to afford the ability to detect >90% of mutations [Vidal-Puig and Moller, 1994]. However, the SSCP analysis in this study focused on the coding region of RET and identifying point mutations therein. Alternative changes in the RET gene such as intronic mutations which alter splicing, or promoter mutations cannot be ruled out at present.

No cytogenetic or molecular aberrations were found in the 14 patients analyzed in this study. One patient carried a pericentrically inverted chromosome 2. However, this inversion was also identified in the phenotypically normal father of the index case. Since pericentric inversions, when familial, are not typically associated with congenital anomalies, we consider it unlikely that chromosome 2 would contain a candidate gene for CCHS.

Since no RET mutations were identified in this study, it is apparent that point mutations in the RET coding regions cannot be implicated as a major causative factor of the occurrence of CCHS, or CCHS and HSCR in the described patient population. We cannot exclude the possibility that another gene or genes involved in neural crest cell migration is/are responsible for CCHS. Neural crest cell migration

along with the neural development of the brain in embryogenesis are complex processes in which many signaling cascades and molecular events are involved. Thus, it will be important to continue to identify additional candidate genes in signaling pathways and molecular events leading to neural cell differentiation and development.

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